Photosensitization of Uroporphyrin Augments the Ultraviolet A–Induced Synthesis of Matrix Metalloproteinases in Human Dermal Fibroblasts

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Porphyria cutanea tarda is characterized by severe connective tissue damage in sun-exposed skin. The regulated synthesis and degradation of the extracellular matrix by various matrix metalloproteinases (MMPs) determine its amount and composition within the skin. In this study, we therefore asked whether long-wave ultraviolet irradiation (340–450 nm) in conjunction with uroporphyrin I could modulate the synthesis of MMPs with substrate specificities for dermal (collagens I, III, V; proteoglycans) and basement membrane components (collagens IV, VII; fibronectin; laminin) and whether synthesis of the counteracting tissue inhibitor of metalloproteinases is also affected. After irradiation of uroporphyrin-pretreated fibroblasts, specific mRNAs of MMP-1 and MMP-3 increased concomitantly up to 2.7-fold compared with ultraviolet-irradiated cells and up to 10-fold compared with mock-irradiated or uroporphyrin I–treated controls. In contrast, mRNA levels of tissue inhibitor of metalloproteinases remained unaltered. Similar results were obtained by immunoprecipitation. Gelatin and casein zymography revealed increased proteolytic activity of MMP-2 and MMP-3 in blister fluids of patients with porphyria cutanea tarda, indicating that similar events may occur *in vivo*. Using deuterium oxide as enhancer and sodium azide as quencher of singlet oxygen, we could increase or reduce MMP synthesis, suggesting that singlet oxygen is the major intermediate in the upregulation of MMPs after irradiation of uroporphyrin-pretreated fibroblasts. Taken together, our results show that ultraviolet irradiation alone, and to a greater extent in conjunction with uroporphyrin I, results in an unbalanced synthesis of MMPs that may contribute to the destruction of the dermis and basement membrane, leading to blistering and accelerated photoaging in porphyria cutanea tarda patients.
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Though blister formation has occasionally been reported after therapeutic ultraviolet A (UVA) irradiation and tanning on sunbeds (Farr *et al*, 1988, Murphy *et al*, 1989, Poh-Fitzpatrick and Ellis, 1989, Weiss and Jung, 1990), it occurs more frequently and severely in patients with porphyria cutanea tarda (PCT), preferentially in chronically UV-exposed skin areas. UVA irradiation has earlier been reported to induce matrix-degrading metalloproteinases (Scharffetter *et al*, 1991, Petersen *et al*, 1992, Herrmann *et al*, 1993). This study was designed to investigate whether and to what extent UVA irradiation (340–450 nm) in combination with uroporphyrin I disturbs the balance between matrix metalloproteinases (MMPs) and their major tissue inhibitor of metalloproteinases (TIMP-1). Our data indicate that UVA irradiation in combination with uroporphyrin I coordinately induces interstitial collagenase (MMP-1), 72-kDa type IV collagenase (MMP-2), and stromelysin-1 (MMP-3). This induction is much stronger than the induction by irradiation alone and seems to be mediated mainly by singlet oxygen. The synthesis of TIMP-1, however, remains unaltered.

The enhanced induction of MMPs upon uroporphyrin-induced photosensitization and the altered balance with their inhibitor TIMP-1 may contribute to the accelerated photodamage and the severe blistering in PCT patients. The *in vivo* relevance of our data is further supported by the finding that MMP-2 and MMP-3 activities are substantially enhanced in blister fluids derived from PCT patients.

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Abbreviations: D_2O, deuterium oxide; MMP, matrix metalloproteinase; TIMP-1, tissue inhibitor of metalloproteinases.

MATERIALS AND METHODS

Reagents Uroporphyrin I, sodium azide (NaN_3), and bovine β-casein were obtained from Sigma (Deisenhofen, Germany); deuterium oxide (D_2O) (99.9%) was from Aldrich (Steinheim, Germany). The cDNA clone for TIMP-1 (TIMP p-3.9x; ATCC 59666) was provided by the American Type Culture Collection (Rockville, MD).

Cell Culture Fibroblast cultures were established by outgrowth from skin biopsy specimens of healthy human donors. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Flow, Meckenheim, Germany) supplemented with sodium ascorbate (50 μg per ml), glutamine (300 μg per ml), penicillin (400 U per ml), streptomycin (50 μg per ml), and

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10% fetal bovine serum. Cells were grown on plastic petri dishes in a humidified atmosphere of 5% CO_2 and 95% air at 37°C (Fleischmajer *et al*, 1981).

Preparation of Uroporphyrin I One milligram of crystalline uroporphyrin I (Sigma) was dissolved in 500 μl of 2 N HCl and neutralized with 500 μl of 2 N NaOH. A Sephadex G-10 column (Pharmacia, Freiburg, Germany) was used to remove salt from the solution. The concentration of uroporphyrin was measured with an ACTA C III spectrophotometer (Beckman, Munich, Germany) following established procedures (Varigos *et al*, 1982). Uroporphyrin I was diluted in DMEM and added to the fibroblast cultures at the indicated concentrations for 24 h.

UVA Irradiation The cells were irradiated at a distance of 40 cm by a high-intensity UVA source (UVASUN 3000 equipped with UVASUN safety filters) emitting wavelengths in the 340- to 450-nm range (Mutzhas, Munich, Germany) (Mutzhas *et al*, 1981). The spectral distribution of the UVASUN 3000 source was determined with a Beckman UV 5270 spectral photometer (Beckman, Munich, Germany). The incident dose rate at the surface of the cells was 66 mW/s. Doses were monitored with a combined UVA/UVB ultraviolet meter (Centra-UV-dosimeter; Osram, Munich, Germany) (Lehmann *et al*, 1986). During irradiation, the cells were incubated in phosphate-buffered saline (PBS) and maintained at 37°C in a thermostatically controlled water bath. After irradiation, PBS was replaced by fresh medium with 10% fetal bovine serum, and the cells were incubated for various times.

Determination of Cell Viability Human dermal fibroblasts at a cell density of 3×10^4 were preincubated with uroporphyrin I at various concentrations (4, 8, and 16 μM) for 24 h and were subjected to irradiation at different doses (90, 180, and 360 kJ/m^2). Twenty-four hours after irradiation, [^{3}H]thymidine (1 mCi per ml, 49 mCi/mmol; Amersham, Braunschweig, Germany) was added to the supplemented DMEM for 10 h. Subsequently, cells were solubilized in 500 μl of solubilization buffer (140 mM NaCl, 20 mM Tris HCl, pH 7.5, 5 mM ethylenediamine tetra-acetic acid, 1% Triton X100). Proteins and DNA were precipitated with 20% trichloroacetic acid (TCA). After three washing steps with 10% TCA, the DNA was redissolved in 3% perchloric acid at 98°C for 30 min. [^{3}H]Thymidine incorporation into the DNA of the fibroblasts was determined by scintillation counting (Tracor Analytic 6892, Frankfurt, Germany). Incorporation of [^{35}S]methionine into proteins was measured to determine total protein synthesis. Fibroblast monolayer cultures were exposed to irradiation. Ten hours after irradiation, cells were washed three times with PBS and were then incubated in methionine-free DMEM without fetal bovine serum for 45 min. Subsequently, 25 μCi of [^{35}S]methionine (specific activity, 1000 Ci/mmol; Amersham) was added per ml. After labeling for 5 h, the reaction was stopped by addition of 0.1 volume of DMEM supplemented with 2 mM methionine and incubation for 45 min. Fibroblasts were lysed in 500 μl of solubilization buffer. Proteins were precipitated by 20% TCA, washed three times with 10% TCA, and redissolved in 0.05 N NaOH. Radioactivity was measured in a scintillation counter. Twenty-four hours after irradiation, fibroblast morphology was monitored under phase contrast microscopy (Zeiss ID02, Oberkochem, Germany). In addition, fibroblast cell numbers were determined with a hemocytometer.

RNA Extraction and Northern Blot Analysis Total RNA was isolated from fibroblast monolayer cultures and analyzed by northern blot transfer hybridization, as published elsewhere (Sambrook *et al*, 1989; Scharffetter *et al*, 1991). For hybridization experiments, the following cDNA fragments were labeled: a 920-bp fragment of the clone K4 corresponding to the 3′ terminal end of the coding sequence and the 3′ untranslated part of MMP-1 mRNA (Angel *et al*, 1986), a 450-bp cDNA fragment of human β-actin (Gunning *et al*, 1983), a 160-bp *Eco*I/*Xba*I cDNA fragment of human MMP-3 (Sirum and Brinckerhoff, 1989), and a 4.05-kb *Xba*I fragment of the genomic clone TIMP-1 (Willard *et al*, 1989).

Immunoprecipitation Immunoprecipitation was performed as described elsewhere (Herrmann *et al*, 1993), with slight modifications. Briefly, 10 h after irradiation, confluent fibroblast monolayer cultures were washed three times with PBS and placed in methionine-free DMEM without serum for 45 min to deplete the intracellular pool of methionine. Subsequently, 10 ml of methionine-free DMEM supplemented with 0.25 mCi of [^{35}S]methionine (25 μCi per ml, 1000 Ci/mmol; Amersham) was added for 5 h. The reaction was stopped by incubation of cells with 0.1 volume of DMEM containing 2 mM methionine for 45 min. Samples of the culture medium of 750 μl were removed at 16.5 h after irradiation and subjected to immunoprecipitation using antisera monospecific for MMP-1 (Stricklin *et al*, 1978), MMP-2 (Vartio *et al*, 1981), MMP-3 (Mitchell *et al*, 1993), and TIMP-1 (Stricklin and Welgus, 1983). For immunoprecipitation, 450 μl of a mixture containing 0.5 M arginine hydrochloride, pH 8.0, 50 μl of 1 M Tris HCl buffer, pH 8.6, 30 μl of 0.2 M ethylenediamine tetraacetic acid, 50 μl of 20% (v/v) Triton X100 in water, and 20 μl of antiserum were added to samples of culture medium and incubated overnight at room temperature. Immune complexes were isolated by adding 40 μl of protein A–Sepharose (Sigma). The Sepharose beads were washed four times with 500 μl of 0.1 M Tris HCl buffer, pH 8.6, supplemented with 250 mM arginine and 1% Triton X100, and washed twice in PBS. The antigen–antibody complexes were dissociated by boiling in 40 μl of sample buffer containing 20% glycerol, 10% β-mercaptoethanol, 4% sodium dodecyl sulfate, 0.125 M Tris, pH 6.8, and 0.002% bromophenol blue, and samples were loaded onto 10% polyacrylamide gels (Laemmli, 1970). Immunoprecipitated MMPs and TIMP-1 were visualized by fluorography.

Zymography Gelatin and casein zymography were used to determine the proteolytic activity of MMP-2 and MMP-3. Blister fluids were obtained by aspiration under sterile conditions. The total protein content was measured according to established methods (Lowry *et al*, 1951), and equal amounts of protein (120 μg/lane) were used for zymography. Gelatin at a final concentration of 2 mg per ml and β-casein at a final concentration of 0.5 mg per ml were included in the running gel instead of urea. Samples were loaded unboiled, and gel electrophoresis was carried out at 4°C. After electrophoresis, the gelatin gels were washed twice in 50 mM Tris HCl, pH 7.6, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, and 2.5% Triton X100 (v/v) for 15 min; the casein gels were washed twice in 50 mM Tris HCl, pH 7.5, and 2.5% Triton X100 for 30 min. Subsequently, gels were rinsed for 15 min in the above-mentioned buffers without Triton X100. Gelatin gels were incubated in 50 mM Tris HCl, pH 7.6, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 1 mM aminophenylmercuric acetate, 1% Triton X100, and 0.02% NaN_3 at 37°C for 10 h (Hibbs *et al*, 1985), whereas casein gels were incubated in 50 mM Tris HCl, pH 7.5, 0.15 M NaCl, 10 mM $CaCl_2$, 1 μM ZnCl, 0.1% Triton X100, and 0.02% NaN_3 at 37°C for 48 h. The gels were stained with Coomassie brilliant blue and destained as described elsewhere (Hibbs *et al*, 1985, Fernandez-Resa *et al*, 1995). Enzymatic activity was visualized by negative staining.

RESULTS

Determination of Cell Viability To establish experimental conditions under which cell viability is not markedly altered, we incubated fibroblasts with various concentrations of uroporphyrin I and irradiated them with different UVA doses, and measured [^{3}H]thymidine incorporation. Preincubation with uroporphyrin I without irradiation did not alter [^{3}H]thymidine incorporation into DNA, but incorporation was markedly reduced after combined treatment of fibroblasts at uroporphyrin I concentrations greater than 8 μM and irradiation doses greater than 180 kJ/m^2. Preincubation at a uroporphyrin I concentration of 16 μM and subsequent irradiation at a dose of 360 kJ/m^2 resulted in a complete loss of DNA synthesis. In contrast, pretreatment of fibroblasts with uroporphyrin I at a concentration of 8 μM and irradiation at a dose of 180 kJ/m^2 resulted in only a 35% decrease in DNA synthesis **(Fig 1)**. Furthermore, preincubation with uroporphyrin I at 8 μM and irradiation at 180 kJ/m^2 changed neither the total protein synthesis as determined by [^{35}S]methionine incorporation nor cell numbers compared with the mock-irradiated control **(Table I)**. Cell morphology was also not altered (data not shown). Accordingly, subsequent experiments were carried out using a uroporphyrin I concentration of 8 μM and an irradiation dose of 180 kJ/m^2.

Earlier and Stronger Induction of MMP-1 and MMP-3 mRNA After Combined Treatment of Human Dermal Fibroblasts With Uroporphyrin I and Irradiation Compared With UVA Irradiation Alone To study the combined effect of uroporphyrin I and irradiation (340–450 nm) on the induction of MMP-1, MMP-3, and TIMP-1, we isolated total RNA at different times after irradiation and performed northern blot analysis. Low constitutive expression of MMP-1, MMP-3, and TIMP-1 mRNA was detected in mock-irradiated cells and in fibroblasts that had been treated only with uroporphyrin I. After irradiation, MMP-1 and MMP-3 mRNAs were 2.4-fold and 3.7-fold induced, respectively, compared with the mock-irradiated controls, as described previously (Scharffetter *et al*, 1991, Herrmann *et al*, 1993) **(Figs 2, 3)**. An initial increase in MMP-1 and MMP-3 steady-state mRNA levels was detected at 12 h after irradiation **(Figs 2, 3)**. Combined treatment of fibroblasts with uroporphyrin I and irradiation led to

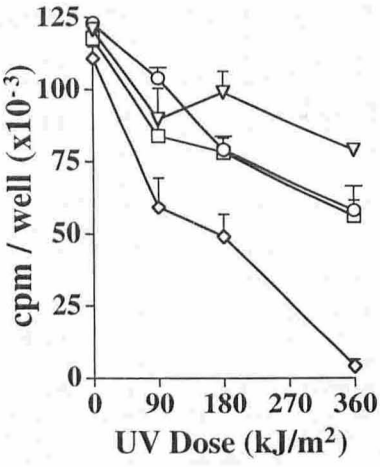

Figure 1. [^{3}H]Thymidine incorporation decreases after treatment of fibroblasts with uroporphyrin I and irradiation (340–450 nm). Human dermal fibroblasts were preincubated with various concentrations of uroporphyrin I (▽, 0 μM; ○, 4 μM; □, 8 μM; ◇, 16 μM) and irradiated at different doses (90, 180, or 360 kJ/m^2). Twenty-four hours after irradiation, [^{3}H]thymidine incorporation into the DNA was determined as described in *Materials and Methods*. Three independent experiments were performed in quadruplicate (mean ± SD).

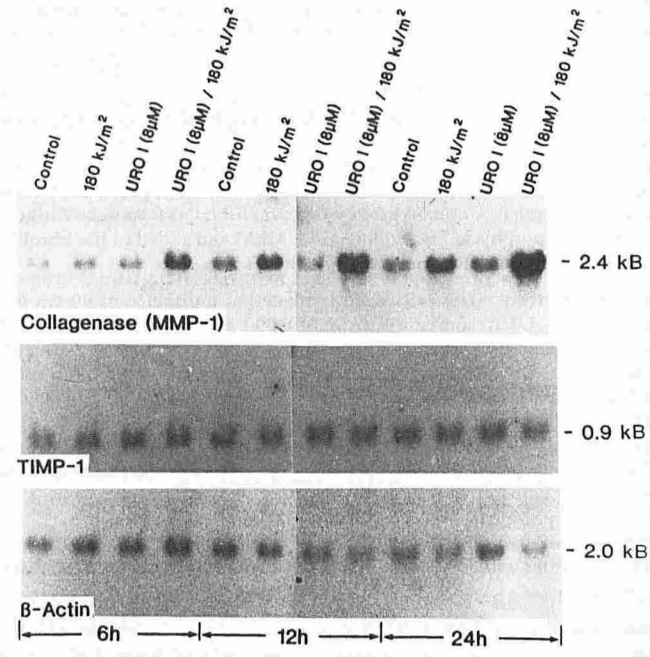

Figure 2. Combined treatment of fibroblasts with uroporphyrin I and UVA augments UVA-induced synthesis of MMP-1. Total cellular RNA was isolated at 6, 12, and 24 h after preincubation with uroporphyrin I at 8 μM and/or irradiation at a dose of 180 kJ/m^2 using the UVASUN 3000 source, as described in *Materials and Methods*. Total RNA was separated by gel electrophoresis under denaturing conditions and blotted onto nitrocellulose. After sequential hybridization with probes specific for human MMP-1, TIMP-1, and β-actin, the filter was processed for autoradiography. The northern blot presented here shows representative data that were reproduced in three independent experiments.

an earlier induction of MMP-1 and MMP-3 mRNA, at 6 h after irradiation **(Figs 2, 3)**. Furthermore, the increase was much greater at 12 and 24 h after irradiation of uroporphyrin I–pretreated fibroblasts than after irradiation of fibroblasts receiving UV alone.

Densitometric evaluation of northern blots with RNA derived from fibroblasts that had undergone treatment with uroporphyrin I and irradiation showed 4.4-fold and 10.1-fold increases in the ratios of MMP-1/β-actin and MMP-3/β-actin compared with the mock-irradiated controls **(Table II)**. In addition, the densitometrically established ratios of MMP-1/β-actin and MMP-3/β-actin of northern blots with RNA derived from fibroblasts after combined treatment with uroporphyrin I and irradiation were 1.8-fold and 2.7-fold increased, respectively, compared with the ratios obtained from fibroblasts receiving irradiation alone. Interestingly, the TIMP-1/β-actin ratio remained unaltered under all experimental conditions **(Fig 3, Table II)**.

Singlet Oxygen Is the Major Intermediate in the Upregulation of MMP-1 mRNA After Combined Uroporphyrin I and UVA Treatment In the presence of D_2O, the lifetime of singlet oxygen is enhanced (Lindig *et al*, 1980), thus increasing the cellular response to singlet oxygen, whereas NaN_3 has been shown to be a potent singlet oxygen quencher (Wilkinson and Brummer, 1991). Singlet oxygen is an important intermediate in the UVA-mediated induction of MMP-1 mRNA (Wlaschek *et al*, 1995). To elucidate further the role of singlet oxygen in the enhanced induction of MMP-1 mRNA after combined uroporphyrin I preincubation and irradiation, we subjected uroporphyrin-pretreated fibroblasts to

Table I. [^{35}S]Methionine Incorporation and Cell Number Are Not Affected After Preincubation With Uroporphyrin I and/or Irradiation (340–450 nm)

Experimental Group	[^{35}S]Methionine Incorporation[a]	Cell Number[b]
Control	1.9 ± 0.4	1.6 ± 0.1
URO I (8 μM)	2.0 ± 0.2	1.6 ± 0.1
180 kJ/m^2	1.8 ± 0.2	1.6 ± 0.1
180 kJ/m^2 + URO I (8 μM)	1.9 ± 0.1	1.6 ± 0.2

[a] Fibroblasts had been treated with uroporphyrin I at 8 μM and/or irradiation at a dose of 180 kJ/m^2. [^{35}S]Methionine incorporation was measured as described in *Materials and Methods*. Results are mean ± SD, cpm × 10^{-5}/10 mm diameter well.

[b] Twenty-four hours after irradiation, cell numbers were counted as described in *Materials and Methods*. Three independent experiments were performed in triplicate and expressed as mean ± SD, cell number × 10^{-5}/35 mm diameter well.

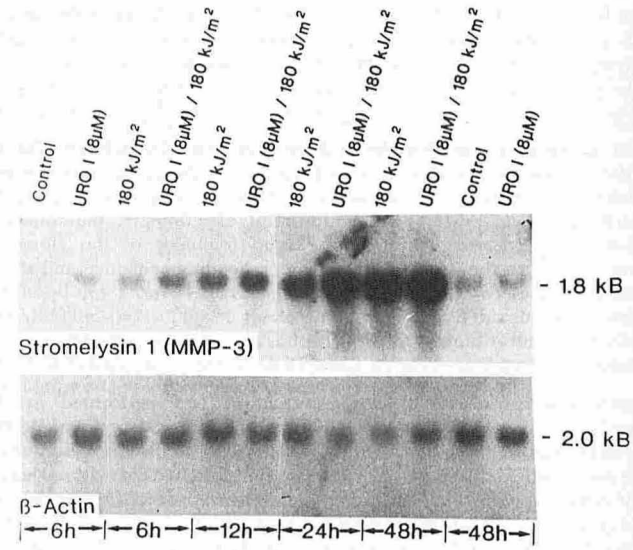

Figure 3. Combined treatment of fibroblasts with uroporphyrin I and UVA augments UVA-induced synthesis of MMP-3. Total RNA was isolated at 6, 12, 24, and 48 h after treatment, as indicated. The experimental design was similar to that described in the legend to **Fig 2**. Sequential hybridization was performed with cDNA probes for human MMP-3 and β-actin.

Table II. Strongest Induction of MMP-1 and MMP-3 mRNA Levels After Combined Treatment of Fibroblasts With Uroporphyrin I and UVA Irradiation[a]

	Control	URO I (8 μM)	180 kJ/m²	URO I (8 μM) + 180 kJ/m²
MMP-1 (collagenase)	1	0.9	2.4	4.4
MMP-3 (stromelysin-1)	1	0.9	3.7	10.1
TIMP-1	1	1	1	1

[a] Data were generated by densitometric analysis and represent fold increase over control at 24 h after irradiation. Experiments were performed in triplicate, SD <5%. Densitometric data were standardized to β-actin.

irradiation in the presence of 95% D_2O or 20 mM NaN_3 in PBS. Neither of these conditions affected cell morphology or cell numbers 24 h after irradiation. Irradiation of uroporphyrin-pretreated fibroblasts in the presence of D_2O, however, resulted in a much greater increase in MMP-1 mRNA than irradiation of uroporphyrin-treated fibroblasts in the presence of H_2O **(Table III)**.

UVA irradiation of fibroblasts in the presence of 20 mM NaN_3, an efficient quencher of singlet oxygen, almost completely abolished the enhanced induction of MMP-1 mRNA after preincubation of fibroblasts with uroporphyrin I and subsequent irradiation **(Table III)**. These findings indicate that singlet oxygen is the major intermediate in the signaling cascade of UV-induced photosensitization of uroporphyrin, finally leading to the enhanced induction of MMP-1 mRNA.

Combined Treatment of Fibroblasts With Uroporphyrin I and UVA Augments UVA-Induced Biosynthesis and Secretion of MMP-1, MMP-2, and MMP-3 To monitor the biosynthesis and secretion of defined MMPs and TIMP-1, we subjected supernatants of fibroblast cultures to immunoprecipitation using affinity-purified antibodies against MMP-1, MMP-2, MMP-3, or TIMP-1. As reported earlier (Herrmann *et al*, 1993), a 2-fold induction of MMP-1, MMP-2, and MMP-3 was found after irradiation (340–450 nm) compared with mock-irradiated controls. Combined treatment of fibroblasts with uroporphyrin I and irradiation resulted in an even greater increase in MMP-1, MMP-2, and MMP-3, up to 4-fold compared with the mock-irradiated controls **(Fig 4)**. Preincubation with uroporphyrin I alone did not affect the amount of MMPs precipitated from the supernatants. Furthermore, after preincubation with uroporphyrin I with or without irradiation, TIMP-1 remained at the same basal level as in the mock-irradiated controls **(Fig 4)**.

Increased MMP-2 and MMP-3 Activity in Blister Fluids From PCT Patients Because MMP-2 and MMP-3 can specifically degrade basement membrane proteins of the dermoepidermal junction (Matrisian, 1992), we monitored the proteolytic activities in the blister fluids of five PCT patients by gelatin and casein zymography. Blister fluids from one patient with bullous pemphigoid, one patient with epidermolysis bullosa Dowling-Meara, and one patient with blisters from insect bites served as controls.

Table III. D_2O and NaN_3 Modulate the MMP-1 mRNA Level After Combined Treatment of Fibroblasts With Uroporphyrin I and UVA Irradiation[a]

MMP-1	H_2O	D_2O	NaN_3 (20 mM)
Control	1	1	1
URO I (8 μM)	1	1	1
180 kJ/m²	2.4	3.5	1.5
URO I (8 μM) + 180 kJ/m²	4.4	7	1.6

[a] Data were generated by densitometric analysis and represent fold increase over control at 24 h after irradiation. Experiments were performed in triplicate, SD <10%. Densitometric data were standardized to β-actin.

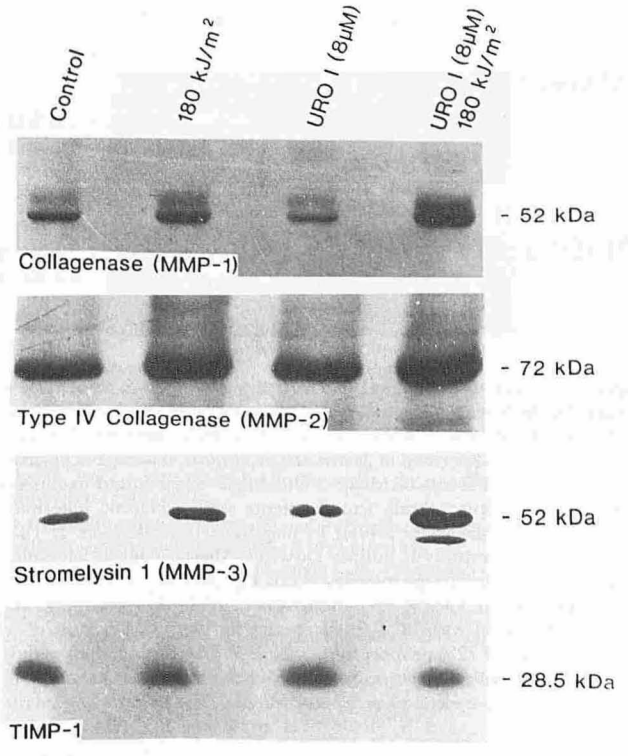

Figure 4. Combined treatment of fibroblasts with uroporphyrin I and UVA augments UVA-induced synthesis and secretion of MMP-1, MMP-2, and MMP-3. Ten hours after irradiation with the UVASUN 3000 source (180 kJ/m²), fibroblasts were transferred to methionine-free medium for 45 min and subsequently to fresh methionine-free medium containing [^{35}S]methionine. After a 5-h incubation at 37°C, the culture medium was harvested and subjected to radioimmunoprecipitation using polyclonal, monospecific antibodies for MMP-1, MMP-2, MMP-3, or TIMP-1, as described in *Materials and Methods*. Different MMPs and TIMP-1 were visualized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and fluorography.

Both the inactive 72-kDa and the active 66-kDa forms of the type IV collagenase (MMP-2) and the inactive 52-kDa and the active 46-kDa forms of stromelysin 1 (MMP-3) were detected in all blister fluids derived from PCT patients. Barely detectable amounts of the inactive 72-kDa and 52-kDa forms were also present in the blister fluids of patients with epidermolysis bullosa Dowling-Meara, bullous pemphigoid, and insect bite blisters. Representative gelatin and casein zymograms of the blister fluids from two PCT patients are shown in **Fig 5**.

DISCUSSION

The development of connective tissue damage exclusively in sun-exposed skin of PCT patients and its improvement after therapeutic reduction of porphyrin concentrations favor the view that uroporphyrin-induced photosensitization is involved in the development of skin lesions. UVA and UVB induction of MMPs has been reported earlier (Stein *et al*, 1989, Scharffetter *et al*, 1991, Petersen *et al*, 1992, Herrmann *et al*, 1993). We provide evidence here that the photodynamic injury of the connective tissue is mediated by an even more dramatic induction of distinct matrix-degrading metalloproteinases with substrate specificities for basement membrane and dermal connective tissue proteins. Accordingly, the induction of MMPs after combined treatment of fibroblasts with irradiation (340–450 nm) and uroporphyrin I is much greater than the induction by irradiation alone. After combined treatment with uroporphyrin I and irradiation, both MMP-1 and MMP-3 steady-state mRNA levels are increased up to 4.4-fold or 10.1-fold, respectively, with identical induction kinetics. Com-

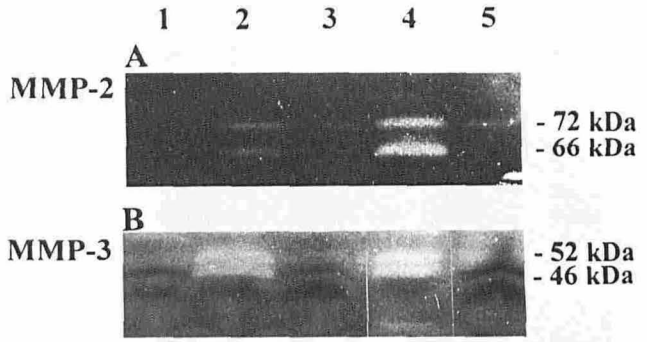

Figure 5. Increased proteolytic activity of MMP-2 and MMP-3 in blister fluids from PCT patients. Gelatin *(A)* and casein *(B)* zymograms of representative blister fluids obtained from PCT patients. Zymography was performed as described in *Materials and Methods*. The active 66-kDa and the active 46-kDa forms of MMP-2 and MMP-3 are found exclusively in PCT patients. Blister fluids from patients with different inherited and acquired bullous disorders served as negative controls. *Lane 1*, 18-y-old woman with epidermolysis bullosa Dowling-Meara. *Lane 2*, 54-y-old man with PCT (total urinary porphyrins: 5778 μg/dl with a relative amount of 52% uroporphyrin). *Lane 3*, 75-y-old woman with bullous pemphigoid. *Lane 4*, 52-y-old woman with PCT (total urinary porphyrins: 6025 μg/dl with a relative amount of 72% uroporphyrin). *Lane 5*, 70-y-old woman with insect bite blisters. Numbers *(right)* indicate molecular weight in kDa.

mon characteristics in the promotor region of the MMP-1 and MMP-3 genes and identical transcription factors may explain their coordinated regulation (Angel *et al*, 1987, Auble and Brinckerhoff, 1991, Wasylyk *et al*, 1991).

MMPs are regulated not only at the level of transcription, but also extracellularly (Docherty *et al*, 1992). Using specific antibodies against various MMPs for immunoprecipitation of cell culture supernatants, we have shown that strong induction of MMP-1, MMP-2, and MMP-3 occurs after combined treatment of fibroblasts with uroporphyrin I and UV irradiation, thus indicating that MMP-1, MMP-2, and MMP-3 proteins are synthesized and actively secreted by fibroblasts.

Our results indicate that synthesis of the major inhibitor of MMPs, TIMP-1, was not affected by the combined treatment of fibroblasts with uroporphyrin I and irradiation. The imbalance between MMPs and TIMP-1 synthesis may enhance connective tissue degradation and lead to the blistering and photoaging that occur in PCT patients (Feldaker *et al*, 1955, Cormane *et al*, 1971, Epstein *et al*, 1973, Wolff *et al*, 1982, Chen *et al*, 1986, Uitto, 1986). Furthermore, increased proteolytic activities of different MMPs were also found in the blister fluids derived from PCT patients, indicating that similar events may occur *in vivo*. In conjunction with the finding that pretreatment of fibroblasts with uroporphyrin I alone did not affect MMP synthesis, and with the clinical observation that skin lesions in PCT patients occur only in sun-exposed skin (Feldaker *et al*, 1955, Cormane *et al*, 1971, Epstein *et al*, 1973), these data support the concept that uroporphyrin-induced photosensitization is responsible for the development of the connective tissue damage. After irradiation, uroporphyrin, like other porphyrins, is photoexcited to a metastable triplet state with subsequent formation of reactive oxygen species, among them singlet oxygen (Poh-Fitzpatrick, 1986, Carraro and Pathak, 1988). In published studies, singlet oxygen generated in a dark reaction by thermal dissociation of the endoperoxide $NDPO_2$ (3,3′-(1,4-naphthylidene) dipropionate) and after UVA irradiation elicited an increase in MMP-1 mRNA and protein levels (Scharffetter-Kochanek *et al*, 1993, Wlaschek *et al*, 1995). Using singlet oxygen enhancer and quencher, we provide evidence that singlet oxygen is indeed the major intermediate in the induction of MMP-1 mRNA after combined treatment with uroporphyrin I and irradiation.

Recent evidence from our laboratory indicates that singlet oxygen also precedes and induces the synthesis of cytokines that mediate the induction of MMP-1 mRNA (Wlaschek *et al*, 1993, 1994, 1995). Data by Devary *et al* (1992) have furthermore indicated that activation of cell membrane–associated Src tyrosine kinases and HaRas small guanosine-binding proteins occurs within minutes after UVC exposure, indicating that the UV response is initiated at or near the plasma membrane. This leads to the activation of nuclear transcription factors, among them activator-protein 1, which enhances MMP-1 and MMP-3 gene transcription. Though these authors have postulated that oxidative stress initiates the UV response, they did not provide experimental data to support this view. Here, we show indirectly that singlet oxygen is responsible for the enhanced induction of various MMPs upon porphyrin-induced photosensitization and thus extend previously published data from this laboratory (Wlaschek *et al*, 1995). Using singlet oxygen quencher or enhancer, we earlier provided indirect evidence that UVA-induced synthesis of MMP-1 is at least partly mediated by singlet oxygen (Wlaschek *et al*, 1995).

Our findings may also help to explain the molecular basis for the recently reported successful treatment of fibrotic skin disorders with 8-methoxypsoralen and UVA irradiation (Kerscher *et al*, 1994, Scharffetter-Kochanek *et al*, 1995). Fibrosis in localized scleroderma and pansclerotic disabling morphea of children is known to be associated with an increase in collagen synthesis and deposition (Krieg *et al*, 1988, Scharffetter *et al*, 1988). Based on our studies, we suspect that 8-methoxypsoralen and UVA irradiation, like uroporphyrin-induced photosensitization, may induce MMP-1 and, by breaking down interstitial collagen, may result in clinical resolution of the sclerotic plaques in scleroderma. In support of this idea, 8-methoxypsoralen is a potent photosensitizer (Joshi and Pathak, 1983), and recent results from our laboratory provided evidence that combined exposure of fibroblasts to 8-methoxypsoralen and UVA irradiation induced a substantial and protracted increase in MMP-1 mRNA in human dermal fibroblasts *in vitro* (Herrmann *et al*, unpublished data).

Taken together, our data show that porphyrin-induced photosensitization results in substantially unbalanced synthesis of MMPs and of their inhibitor TIMP-1 and thus may contribute to the dissolution of dermal and basement membrane components, leading eventually to blistering and photoaging. The enhanced expression of MMPs after porphyrin-induced photosensitization might explain the common clinical observation that photoaging occurs at an earlier age and blistering is more severe in PCT patients than in patients exposed exclusively to therapeutic UVA irradiation.

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